

## Phylogeographic history of the Japanese Alpine Ringlet *Erebia niphonica* (Lepidoptera, Nymphalidae): Fragmentation and secondary contact

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**Abstract** There have been some previous studies about when and through which route the alpine organisms ranging in the Japanese islands migrated from the continent, and how their distribution range has changed within the islands while adapting to the climate changes in the Quaternary. There are studies about alpine plants based on recent molecular information. A scenario has been proposed that there are two lineages. One is the lineage that had relict distribution during interglacials. The other is the lineage that migrated into the Japanese islands during later glacial periods. On the other hand, animals have mobility and are able to move around following environmental changes such as climate changes. Therefore, there is a possibility of having a different scenario from that of plants. We chose *Erebia niphonica*, and studied the distribution change of alpine organisms in the Japanese islands during the Quaternary. We studied the haplotypes of base sequences of 942 bp in total using *ND5* and *COI*. Samples are 427 individuals from the Japanese islands, Mongolia and Russia. We inferred past historical events by analyzing the haplotypes using the Nested Clade Phylogeographical Analysis (NCPA). As a result it has been suggested that the *Erebia niphonica* group went through isolation in more than one refugium in Hokkaido and more than one in Honshu during warm interglacials, and that they fragmented into different lineages within the Japanese islands. Also we specified the secondary contact points which support the NCPA which inferred that range fragmentation and dispersal were repeated.

**Key words** Phylogeography, biogeography, *Erebia niphonica*, nested clade phylogeographical analysis, haplotype, *ND5*, *COI*, Quaternary.

## 1. Introduction

### 1.1 Phylogeographic history of the Japanese islands

There were periods when the Japanese islands [the Japanese Archipelago] were connected with the Asian continent during the glacial periods and served as refugia for animals and plants of continental origin. During the glacial age, many of the arctic and subarctic animals and plants migrated into the Japanese islands. During the interglacial age, distributions of those animals and plants diminished or disappeared (Yasuda and Miyoshi (Eds), 1998; Machida *et al.*, 2003). There have been many studies of fauna based on fossils (Kawamura *et al.*, 1989; Kawamura, 1998), and of flora based on pollen and wood fossils (Sohma and Tsuji, 1988; Suzuki and Takeuti, 1989; Morita, 2000).

The major islands that constitute the Japanese islands are Sakhalin, Hokkaido, Honshu, Shikoku, and Kyushu. They stretch in a row from north to south (Fig. 1). There is the Tartar Strait (=the Mamiya Strait) between the Amur region and Sakhalin, and the Soya Strait between Sakhalin and Hokkaido, and the Tsugaru Strait between Hokkaido and Honshu, and the Tsushima Strait between the Korean Peninsula and Honshu-Kyushu. Of these, the Tartar Strait and the Soya Strait are shallow, and were formed later than 11,000

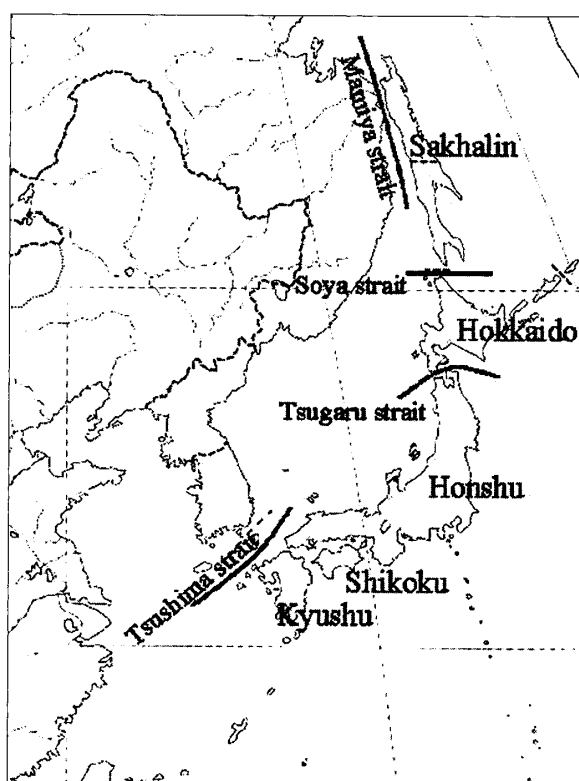


Fig. 1. The Japanese islands. Shows major islands and straits.

years ago. On the other hand, the Tsugaru Strait and the Tsushima Strait are deep. Honshu separated from the Korean Peninsula and Hokkaido and became an island during the interglacial age after the Riss glaciation about 150,000 years ago. It is believed that Honshu has not been connected with the continent since then (Ohshima, 1990, 2000; Matsui *et al.*, 1998). Sakhalin and Hokkaido had been connected with the continent almost continuously during the period of 65,000 years from about 75,000 years ago up to about 10,000 years ago (Ono, 1990). They had been parts of the continent for longer periods of time than the periods when they were isolated islands. Thus, during the glacial period the geographic condition allowed organisms to migrate southward from the continent to Sakhalin and Hokkaido. On the other hand, alpine organisms that now have isolated distribution in the alpine belt of Honshu had expanded their distribution prior to about 150,000 years ago from the continent, and they are the species that were affected by climate changes since then during the interglacial period and the last glacial period.

The high altitude areas of central Honshu and Hokkaido in the Japanese islands have had at least two periods when glaciers developed, and there is mountainous permafrost in some limited areas in the high mountainous areas in Hokkaido and central Honshu even now (Iwata, 2003). Reconstruction studies of ancient flora by pollen analyses (Sakaguchi, 1989; Igarashi *et al.*, 1993; Yamada, 1998; Hoshino, 1998) suggest that the low altitude areas in Hokkaido were the southern limit of taiga during the last glacial period, and that there were forests richer with *Larix gmelinii* than those in the present northern Sakhalin. In the eastern and northern parts of Hokkaido, the distribution of taiga with *Larix gmelinii* as its main plant was divided in such a way that each of coniferous forests, grasslands, wet vegetation and alpine tundra formed segregated niches according to altitude and/or the geographical features. Thus the areas functioned as refugia for many organisms in the Far East (Ono, 1990).

In Honshu there is a large rift zone called Fossa Magna that runs across the island from NNW to SSE at around 138 degrees of east longitude. The effect of the rift zone on the distribution of organisms has been noted biogeographically (Su *et al.*, 1998). However, there has been no study that suggests relevance of the rift zone to phylogeography of alpine organisms. The time of its tectonic formation (it was under the sea up until several millions years ago) was much earlier than the time when the alpine organisms came to the Japanese islands.

Regarding the understanding of the immigration routes of alpine butterflies into the Japanese islands and varying patterns of distribution on the islands, the limitations of regional biogeographic approaches or of studies based on the degree of specialization of external characters have been pointed out (Hiura, 1971, 1977). There were, however, no alternative means of approach. Now, using the new viewpoint of molecular phylogenetics, there have been studies on *Pedicularis chamissonis* (Fujii *et al.*, 1997) and on *Primula cuneifolia* (Fujii *et al.*, 1999). There are also phylogeographic studies on the whole Eurasian populations of Japanese Rock Ptarmigan *Lagopus mutus japonicus* (Baba *et al.*, 2001) and on the Small Tortoiseshell butterfly *Aglais urticae* (Vandewoestijne *et al.*, 2004).

The process of range expansion and contraction of organisms due to climate changes in the Quaternary is well known as an east-west migration in western Eurasia (Hewitt, 1996). But as the geographic characteristics of the north and south extension of the Japanese islands may suggest, the process in the Japanese islands is composed of north-south migration. Each species of alpine plants has two lineages in Honshu, northern and southern, and the boundary is in the northeast of the island (Fujii *et al.*, 1997, 1999). As the primary cause of such a distribution character, a scenario has been suggested whereby the original population remained in the mountainous areas of central Honshu as refugia during the interglacial period and later became the southern lineage, and a new lineage migrated into the Japanese islands during the glacial period after the interglacial period and formed the northern lineage (single refugium model). However, it could be considered to be possible that more than one refugium existed in the Japanese islands because of its complex mountain topography and the micrometeorological environments caused by it. That is to say, there could be a new scenario. As a result of isolation by taking refuge in more than one refugium during the warm periods, genetically differentiated populations have survived till now in more than one lineage by expanding and contracting their distribution areas adapting to later glacial cycles (multiple refugia model). In order to study such a possibility, it is necessary that the subject organisms of the study must have the following characteristics (Schmitt *et al.*, 2006; DeChaine and Martin, 2004). (1) Adapting to environmental changes, they should be able to move to suitable surroundings. (2) Each individual does not usually move around and tends to stay in the maternal population, and they inherit phylogeographical information as a population. (3) They are able to maintain continuous populations even when the areas with inhabitable environment are geographically small. (4) They are currently distributed in wide areas even though sporadic.

Body sizes of insects are relatively small, and they are capable of reproducing themselves as stable populations in relatively small habitats. Butterflies have more advantageous capabilities than plants for maintaining populations under the influence of glacial cycles, such as migrating to more suitable surroundings on their own. Therefore, it is highly possible that there is a historical scenario for alpine butterflies different from that of alpine plants.

## 1.2 Systematics and ecology of the subject species

We have chosen *Erebia nipponica* as the subject species of this study, and we will verify that this species satisfies the aforementioned requirements (1 to 4). The species is found in

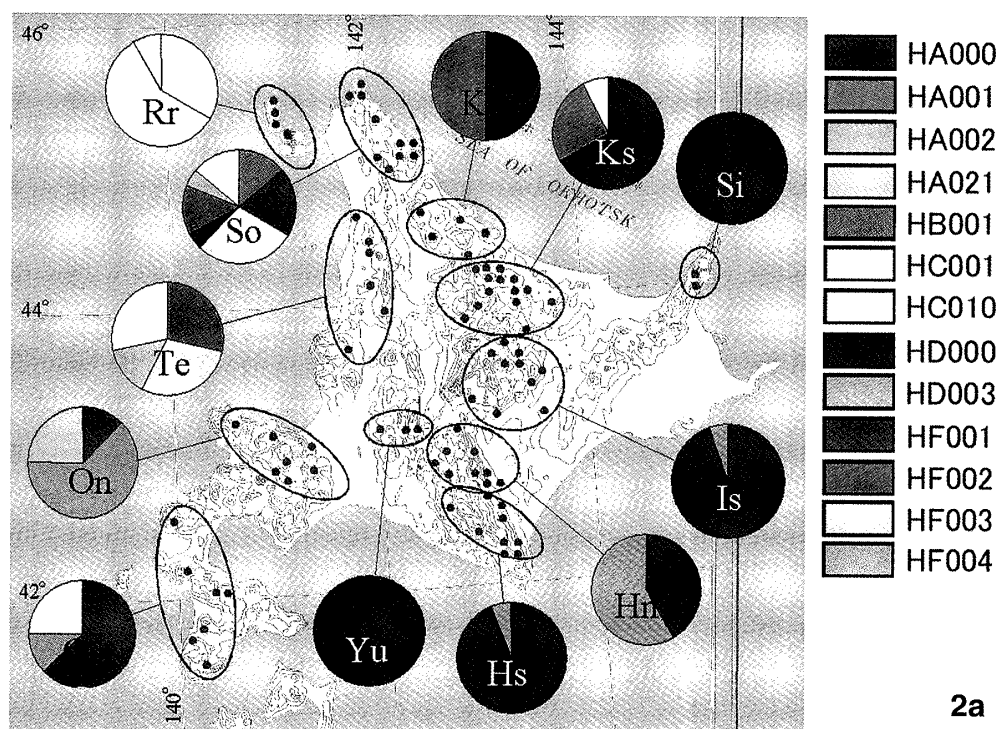
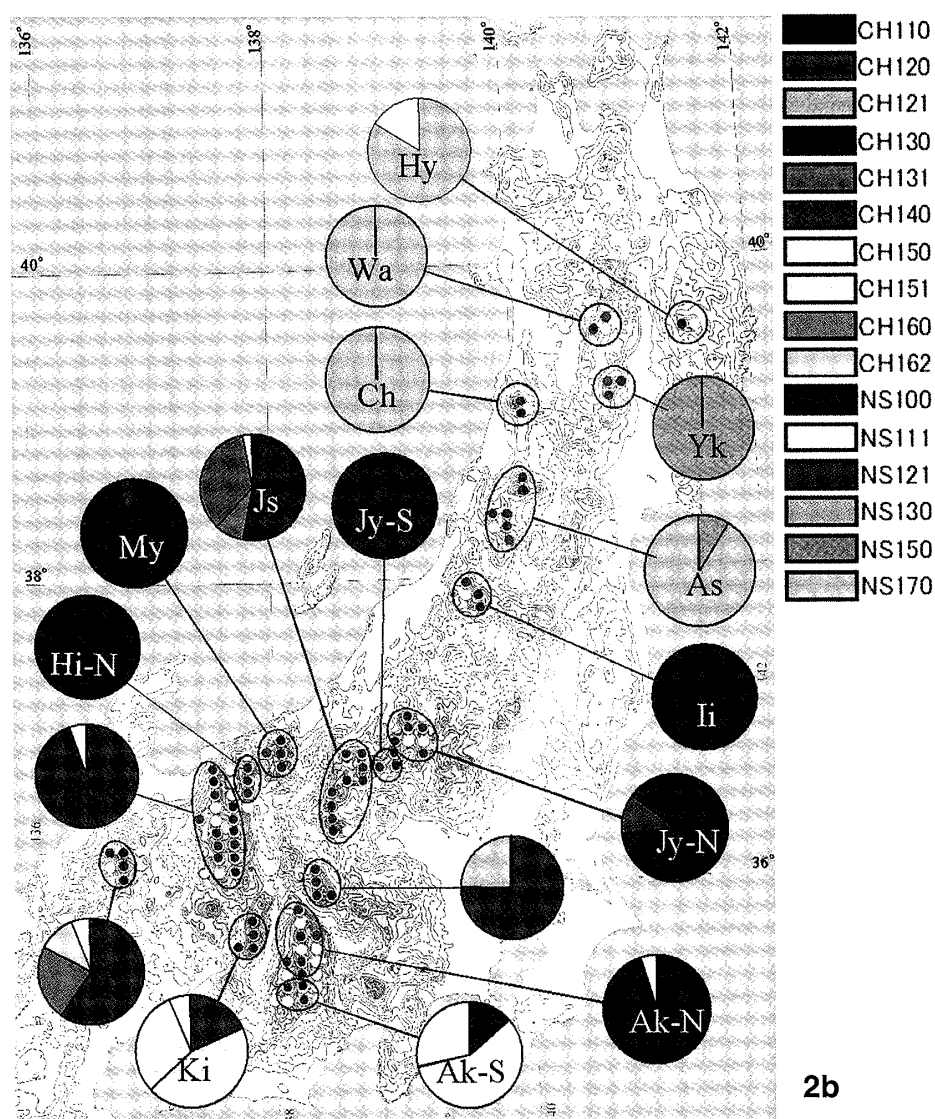


Fig. 2. *Erebia nipponica* distribution ranges and sample sites; (a) Hokkaido, (b) Honshu. Pale green dot denotes the distribution ranges, red dot denotes the sample site. Indication unit is 7.5' longitude  $\times$  5.0' latitude. Pie charts show the proportions of haplotypes within each region. White is private haplotypes restricted in one region. For abbreviations refer to Table 1.

Sakhalin, the southern Kuril Islands (Kunashir Island), Hokkaido, and the alpine areas of Honshu, and can be classified into several subspecies (Fujioka, 1981; Inomata, 1986). In northeastern Eurasia the closely related *Erebia neriene* is distributed. There is much controversy about the taxonomic relationship of *nipponica* and *neriene*. Some taxonomists regard them as geographical variations of the same species (Fujioka, 1981; Asahi *et al.*, 1999), some as different species (Warren, 1936; Inomata, 1986; Gorbunov, 2001), and some regard only the Honshu one as an independent species (Kogure, 1979). In the monograph on the genus *Erebia* (Warren, 1936), these taxa belong to the *aethiops* group together with *E. aethiops* which ranges from Europe to near Lake Baikal in southern Siberia, and *E. alcmena* which is found in the alpine area of central south China. The monophyly of this group has been verified by molecular phylogeny (Sekiguchi *et al.*, 2002). There was a view that *Erebia vidleri*, which is found in northwestern North America and *Erebia nipponica* are closely related species (Layberry *et al.*, 1998), but they are quite far apart based on recent molecular phylogeny (Nakatani *et al.*, in preparation).

*Erebia nipponica* inhabits grasslands along streams in subalpine coniferous forests above 1,000 m above sea level in northeastern Honshu and above 1,500 m in central Honshu, grasslands in sparse forests of *Betula ermanii* near the timberline, and alpine herbaceous and scree grass land above the timberline (3,000–3,050 m maximum) (Nakatani and Kitagawa, 2001). In Hokkaido it ranges widely from near the seashore to the alpine herbaceous zone around 1,600 m above sea level (Takeuchi, 2003). In Sakhalin it occurs in the south of the central part (N51–N52 degree), and ranges from near the seashore to grasslands in the forests (Asahi *et al.*, 1999; Asahi and Kohara, 2004).



As for the larval food plants of *Erebia niphonica*, about 10 species of Gramineae and Cyperaceae have been listed (Fukuda *et al.*, 1984). In Hokkaido, larvae have also been found from *Poa pratensis* and *Festuca rubra* which were brought in and planted on the slopes of woodland trails (Kawata and Kitahara, 1990). This species is a generalist for larval food plants and appears once a year.

The habitats in Honshu consist of relatively small fields isolated by forests, shrubs, or bamboo grass lands, and population size is small (Nakatani and Kitagawa, 2000). There has been no detailed study on the home range size. We have observed that the insects remained still in the thickets under strong wind conditions, and that they had a tendency to land quickly and remain still when forced to fly. It seems that usually they seldom move from the natal site. However, a case has been reported in which a new population started to breed along a new trail which had been opened in a subalpine coniferous forest (Kawata and Kitahara, 1990; Nakatani and Hosoya, 2003). Therefore, females seem to have some migrating habit. In other words, that the process repeats itself whereby a new colony comes into existence at the periphery of distribution ranges, and then becomes extinct as grassland shifts to shrub or bamboo grassland.

Table 1. Distribution of the haplotypes (ND5 plus COI) of *E. nipponica* (columns) from each sampled ranges (rows).

Island	Range	Site name	Latitude Longitude	HB001	HA000	HA001	HA002	HA021	HC001	HC010	HD000	HD003	HF001	HF002	HF003	HF004	NS100	NS130	NS150	NS170	NS111	NS121	CH110	CH120	CH121	CH130	CH131	CH140	CH150	CH151	CH160	CH162	Private	Total	Standard diversity indices
Hokkaido	Rr	Rebun, Rishiri	45.22.21 141.03.30						4	7																						1	12	0.5909±0.1079	
	So	Soya region	45.06.07 142.15.48	3	4			6			1		3			1																3	21	0.8714±0.0454	
	Kn	N Kitami Mts	44.31.39 142.50.36								3			3																		6		0.6000±0.1291	
	Ks	SW Kitami Mts	44.03.03 143.06.40								18		7																			2	27	0.5043±0.0879	
	Is	Ishikari Mts	43.31.35 143.15.13								18	1																				19		0.1053±0.0920	
	Si	Shiretoko reg.	44.03.17 145.06.16								2																					2		0.0000±0.0000	
	Yu	Yubari Mts	43.06.51 142.10.59								8																					8		0.0000±0.0000	
	Hn	N Hidaka Mts	42.57.06 142.42.54								5	7																				12		0.5303±0.0764	
	Hs	S Hidaka Mts	42.39.28 142.51.40								16	1																				17		0.1176±0.1012	
	Te	Teshio Mts	44.30.00 141.53.27										2	2	1																	2	7	0.9048±0.1033	
	On	N Oshima pen.	42.55.51 141.02.25	1	5	2																										8		0.6071±0.1640	
	Os	S Oshima pen.	41.48.48 140.16.26		5	1																										2	8	0.6429±0.1841	
	Honshu	Hy	Hayachine Mts	39.33.28 141.27.37															5														1	6	0.3333±0.2152
Wa		Mahiru Mts	39.34.15 140.45.13															7														7		0.0000±0.0000	
Ch		Chokaisan	39.06.04 140.01.46															5														5		0.0000±0.0000	
Yk		Yakeishi reg.	39.10.33 140.51.21																5													5		0.0000±0.0000	
As		Asahi Mts	38.16.44 139.53.49																	1	10											11		0.1818±0.1436	
Ii		Iide Mts	37.51.42 139.39.38																													10		0.0000±0.0000	
Jy-N		N Joetsu reg.	36.52.51 138.57.40																													41	5	6	0.4390±0.0833



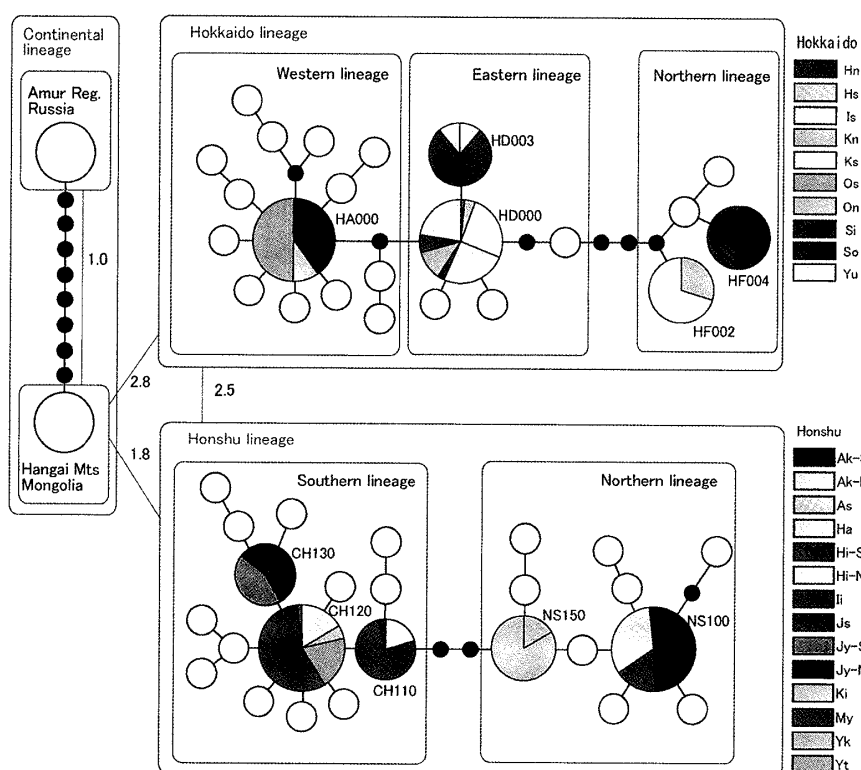


Fig. 3. Statistical parsimony network. Circles denote haplotypes, size of the circle reflects the wideness of distribution ranges in 3 levels, small black circles denote missing or theoretical haplotypes, solid lines connecting each pair of haplotypes represent a single mutational step, regardless of their length. Pie charts show the proportions of regions within each haplotype. Uncolored haplotype was detected in one region only. The haplotypes from the continent are from Khrebet Khekhtsir, Amur region, Russia (N48°15'21", E135°03'57") and Tsenkher Jiguur SPA, Hangai Mts, Mongolia (N47°19'16", E101°39'25"). Genetic distances (%) are calculated in Kimura's 2 parameter method based on *COI* (510 bp).

We can see that the ecological characteristics of *Erebia nipponica* well satisfy the aforementioned necessary conditions for the study of phylogeographic history. Since the goal of this study is to know how the glacial cycles of the Pleistocene affected the genetic diversity of alpine butterflies, it is desirable to collect samples at many sites covering a wide range of geographical distribution (Vila *et al.*, 2005).

## 2. Materials and methods

### 2.1 Specimens and collection of molecular information

We have collected 423 individuals in 289 populations in total, including 147 individuals in 119 populations in Hokkaido and 276 individuals in 170 populations in Honshu. Fig. 2 shows the distribution and sampling sites of *Erebia nipponica* in the Japanese islands with 7.5' longitude  $\times$  5.0' latitude as a unit, and the sampling sites cover approximately 70% of the distribution range. Samples were collected between years 2003 and 2005. Although many samples including males and females are required for reliable statistical analyses, from the standpoint of nature conservation, we have collected only males as samples. In samples from some areas, specimens were included that had been collected, dried and preserved since 1996. We also analyzed 2 individuals of *Erebia neriene* from the Khangai



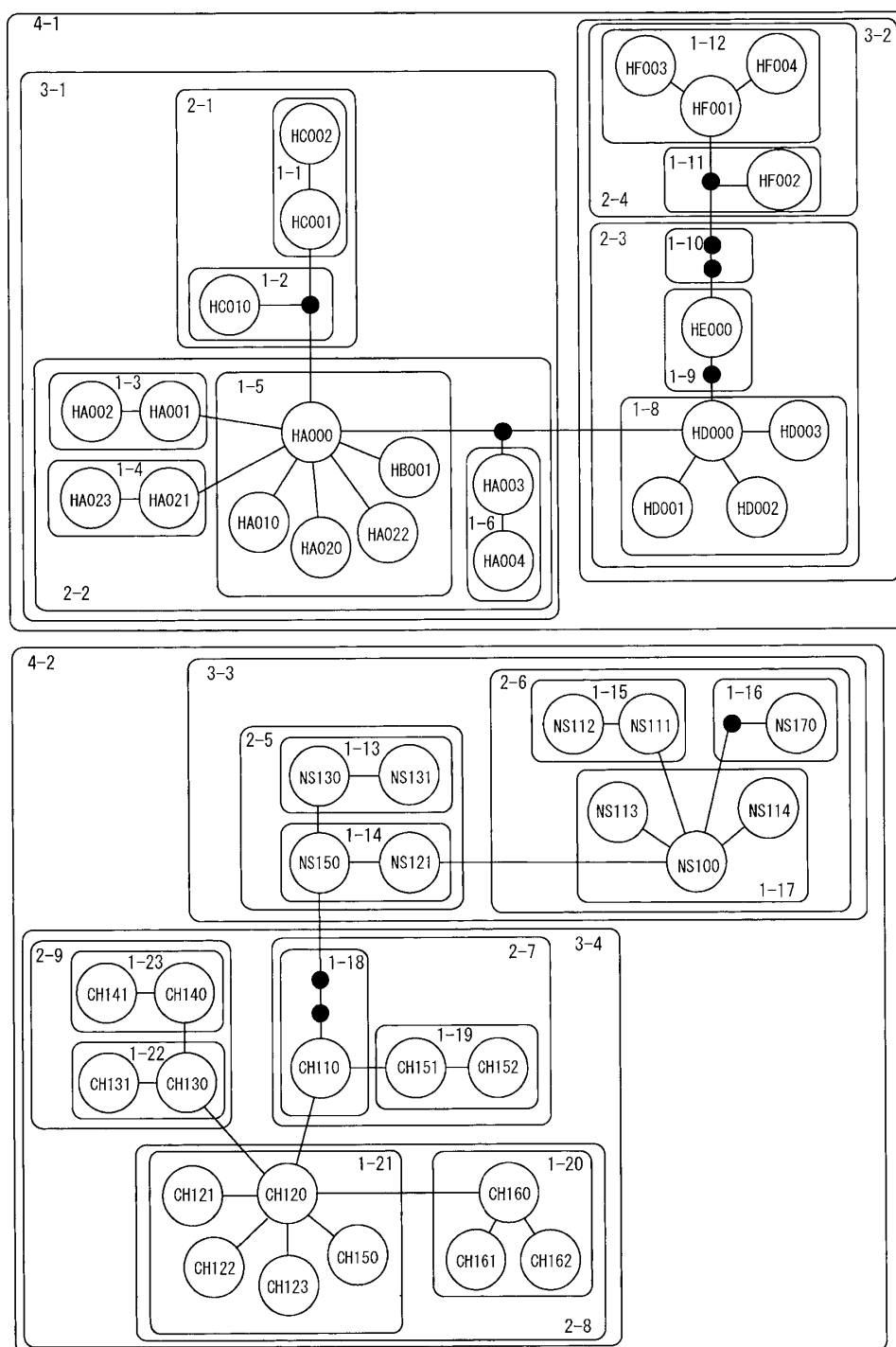


Fig. 4. Haplotype network and associated nested design. All haplotypes are nested in 4th level clade in both islands.

Table 2. AMOVA results for test of genetic subdivision between populations.

## (a) Hokkaido

Source of variation	Variance components	% of variation
Among mountain regions	0.746*	47.48
Among population within mountain regions	0.133	8.45
Within population	0.693*	44.07

## (b) Honshu

Source of variation	Variance components	% of variation
Among mountain regions	1.492*	77.08
Among population within mountain regions	0.165*	8.51
Within population	0.279*	14.41

\*  $P < 0.001$ .

Mountains in Mongolia and 2 from the Amur region in Russia for reference. Each sample was caught with a net, wrapped in paraffin paper, and brought back within two days. The body of the sample without wings was preserved in 99% ethanol and kept at 4°C until DNA was extracted.

For genetic markers, we used a part of the NADH dehydrogenase subunit 5 (*ND5*) and cytochrome oxidase subunit I (*COI*) genes in mitochondrial DNA. DNA was extracted from the body and legs by DNeasy Tissue Extraction Kit (Qiagen). PCR was conducted in a 10  $\mu$ l reaction system by adding DNA (*ca* 40 ng), as recommended by Applied Biosystems reaction constitution. Thermal cycler profile was one cycle at 95°C for 10 min; 25 cycles at 95°C for 30 sec, 45°C for 30 sec, 72°C for 1 min 30 sec, one extension cycle at 72°C for 8 min 30 sec. PCR products were processed after Exo-SAP treatment. The nucleotide sequences of the amplified DNA fragments were determined by direct sequencing method with Big Dye Terminator v1.1 Cycle Sequence Kit. As primers for PCR and direct sequencing, we used V1 (5'-CCT GTT TCT GCT TTA GTT CA-3'), C2 (5'-ATC YTT WGA ATA AAA YCC AGC-3') (Yagi *et al.*, 1999) for *ND5*, and mtD6 (C1-J-1718; 5'-GGA GGA TTT GGA AAT TGA TTA GTT CC-3'), Nancy (C1-N-2191; 5'-CCC GGT AAA ATT AAA ATA TAA ACT TC-3') (Simon *et al.*, 1994) for *COI*. We aligned the nucleotide sequence data with Clustal X (Thompson *et al.*, 1997), and conducted the fragment comparison of 432 bp for *ND5*, 510 bp for *COI*, and 942 bp in total.

## 2.2 Analysis of genetic characteristics of populations

In order to test isolation by distance, we divided the Hokkaido population into 12 groups and the Honshu population into 17 groups (Table 1). We then computed the genetic variance at each hierarchical level within a population, between populations, and between groups based on AMOVA (Excoffier *et al.*, 1992) by software Arlequin 2.0 (Schneider *et al.*, 2000). The number of permutations was set at 10,000 times when computing. Genetic diversity of a population is considered to increase by repeating the process of range fragmentation into local groups and re-merging under climate changes (DeChaine and Martin, 2004). We calculated standard diversity indices by Arlequin 2.0 as the indicator of genetic diversity of the population.

## 2.3 Nested clade phylogeographical analysis

Nested clade phylogeographical analysis (NCPA) (Templeton *et al.*, 1995; Templeton, 1998, 2004) can infer past events that affected the population constituents of a species such as past fragmentation, restricted gene flow and range expansion by combining haplotype

Table 3. Inferences of historical processes.

## (a) Hokkaido

Clade	Steps in inference key	Inferred event	$\chi^2$	P-value
1-5	1-2-3-4-9-No	Allopatric fragmentaion	30.08	0.116
1-8	1-2-3-4-9-No	Allopatric fragmentaion	48.39	0.096
2-2	1-2-3-4-No	Restricted gene flow	48.49	0.047
2-3	1-19-20-No	Inadequate geographical sampling	1.00	0.021
2-4	1-19-No	Allopatric fragmentaion	19.00	0.000
3-1	1-19-No	Allopatric fragmentaion	45.00	0.000
3-2	1-2-11-12-No	Contiguous range expansion	51.98	0.000
4-1	1-19-No	Allopatric fragmentaion	127.68	0.000

## (b) Honshu

Clade	Steps in inference key	Inferred event	$\chi^2$	P-value
1-14	1-19-20-2	Inconclusive outcome*	11.00	0.002
1-21	1-2-3-4-9-No	Allopatric fragmentaion	117.81	0.000
2-5	1-19-No	Allopatric fragmentaion	29.00	0.000
2-6	1-2-11-12-13-Yes	Past fragmentation followed by range expansion	131.95	0.000
2-7	1-19-20-2-11-12-13-Yes	Past fragmentation followed by range expansion	1.00	0.000
2-8	1-2-3-5-6-13-Yes	Past fragmentation followed by range expansion	44.81	0.000
2-9	1-2-3-4-No	Restricted gene flow with isolation by distance	39.44	0.000
3-3	1-2-11-12-No	Contiguous range expansion	82.92	0.000
3-4	1-2-3-5-6-7-Yes	Dispersal but with some long distance dispersal	297.24	0.000
4-2	1-19-No	Allopatric fragmentaion	257.95	0.000

$\chi^2$ -test of geographical association of clades and inferences of biological causes for such association.

The inferences were obtained following the most updated key (Templeton, 2004).

\*Tip/Interior status cannot be determined.

phylogeny and geographical information of its distribution. We inferred topology among haplotypes by using statistical parsimony methods (Templeton *et al.*, 1992) integrated into the TCS program (Clement *et al.*, 2000). The network is connected with 95% reliability that multiple transfers do not occur at any locus. The relationships between the inferred phylogeny and geographic position can be evaluated by comparison of two distances of the within clade distance (Dc) and the nested clade distance (Dn), and the interior-tip contrasts. The significance of the distances defined by NCPA is evaluated using  $\alpha=0.05$ . These evaluations were calculated by the GeoDis Program (Posada *et al.*, 2000). When the evaluation is significantly large (L) or significantly small (S), they indicate that haplotype/clade distribution is significantly widespread or significantly restricted. Significant I-T contrast for both Dc and Dn values denotes a displaced (L) or restricted (S) distribution of lower *versus* higher haplotypes/clades.

### 3. Results

Distribution frequency of haplotypes and sample sites is shown in Table 1. As a result, 22 haplotypes from Hokkaido and 25 haplotypes from Honshu were detected. The number of substitutions of *ND5* is 28 (6.5%) and that of *COI* is 31 (6.1%). The substitution rates of both genetic areas are quite similar. Among 59 variable loci, those at the first codon were 12 (20.3%), the second codon 4 (6.8%), and the third codon 43 (72.9%). Nucleotide frequencies were strongly AT biased as in many other insects (frequencies: A=30.2%, C=13.7%, G=13.4%, T=42.7%). There were 10 nonsynonymous substitutions, and 2 of them appeared in samples both from Hokkaido and from Honshu populations.

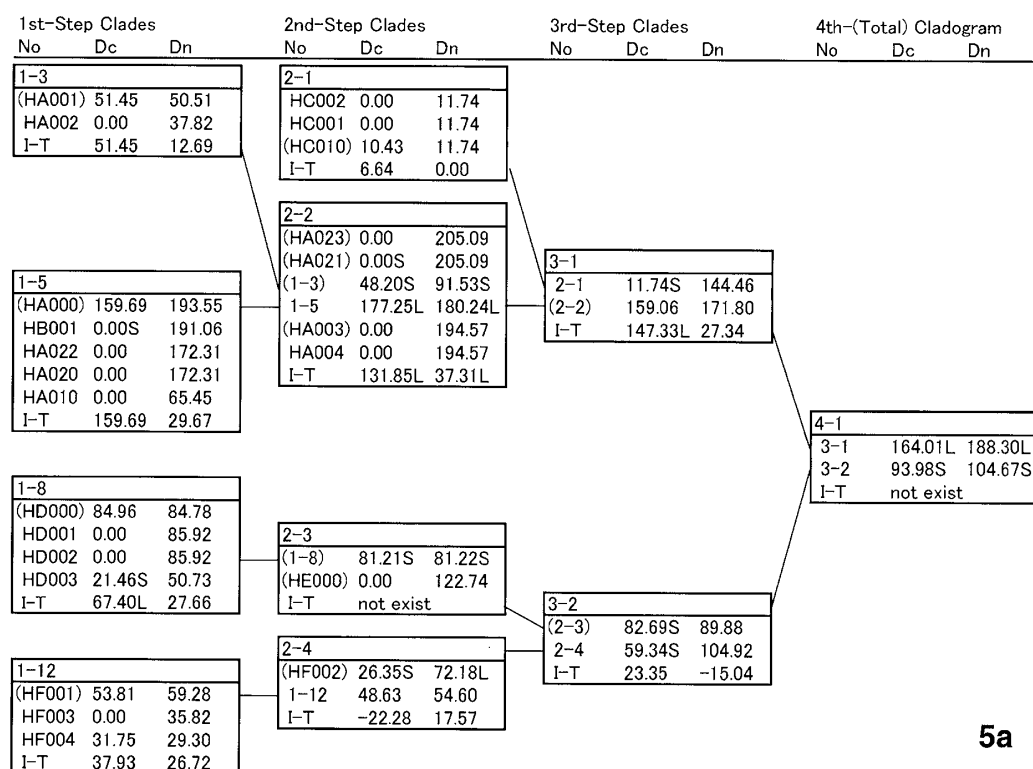


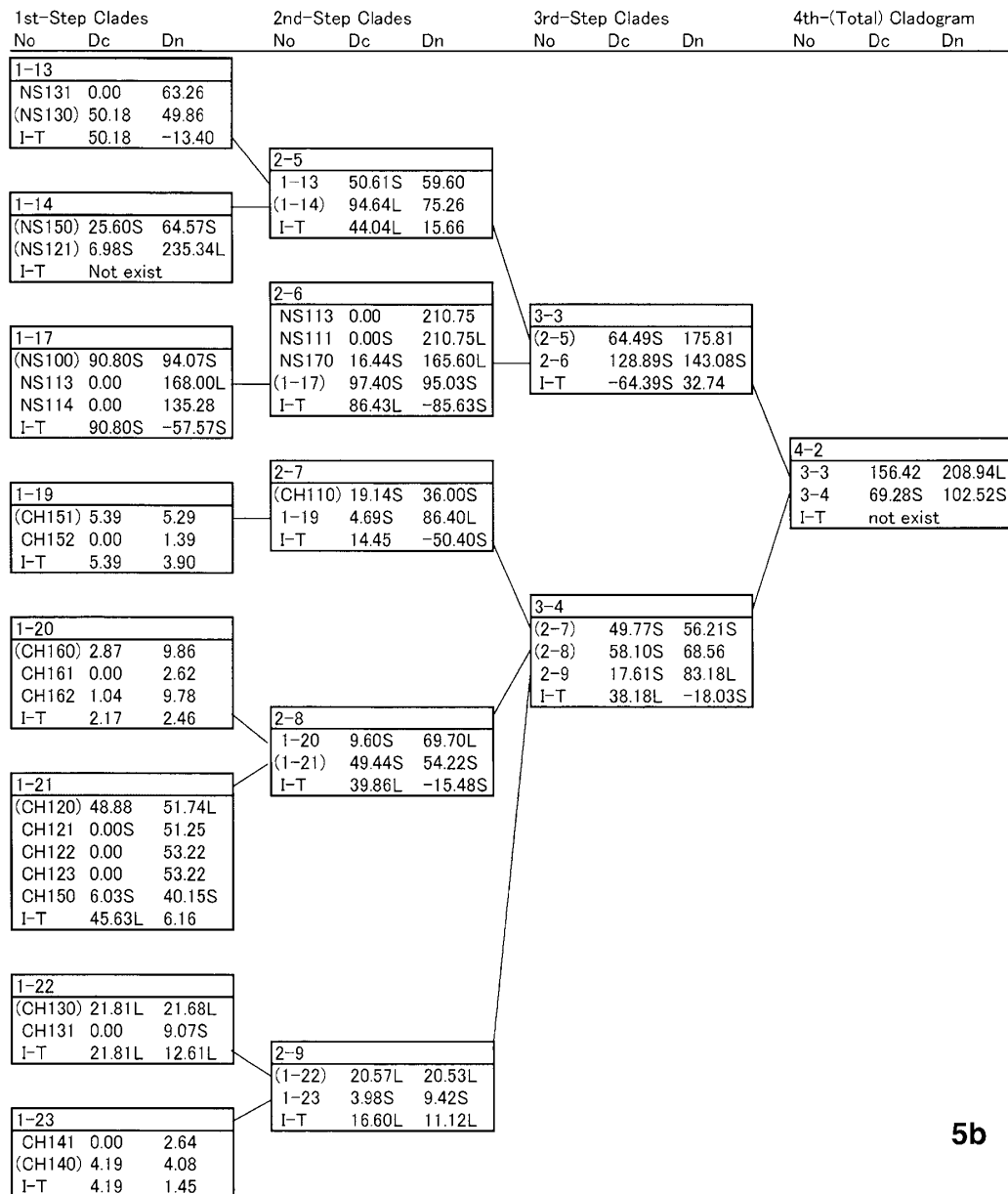
Fig. 5. Analytical results for NCPA with significant geographic structures. Nesting hierarchy proceeds from the most recent 1st level clades on the left to the older clades on the right. Within each clades (boxed), columns indicate haplotypes, one hierarchical lower level clades or interior-tip contrasts, within clades distance (Dc), and nested clades distance (Dn). Individual haplotypes/clades in parentheses are interior. Significantly large and small statistics are denoted by L and S, respectively. (a) Hokkaido. (b) Honshu.

### 3.1 Genetic difference among the continent, Hokkaido, and Honshu populations of *E. nerienne* and *E. nipponica*

Topology of the relationships among haplotypes was inferred using a statistical parsimony method (Fig. 3). Nine missing or theoretical haplotypes were inferred. Due to the number of mutational steps separating the continental, Hokkaido and Honshu haplotypes, they could not be joined to the network with greater than 95% confidence. *COI* (510 bp) fragment comparison indicated that there is about 2.5% difference of substitutions between major haplotypes of the Hokkaido and Honshu populations. Also, between the *E. neriene* population on the continent (collected in the Khangai Mountains, central Mongolia) and the *E. nipponica* population in Hokkaido or that in Honshu, there is about 2.8% and 1.8% difference, respectively. Within the continent, the difference between those taken in the Khangai Mountains and those in the Amur region in Russia is only 1.0%.

### 3.2 Genetic variations

As a result of analysis of variance by AMOVA (Table 2), 48% of molecular variance in Hokkaido and 77% in Honshu were observed between the regions. These results suggest that gene flows are restricted and that there is geographic isolation between the regions. Standard diversity indices were calculated (Table 1). Diversity is very high in the Soya area (So) and in the Teshio Mountains (Te), which are located in the northern part of Hokkaido. In the central area (Is and Yu), diversity is low. In Honshu, diversity is very high in the Kiso



Mountains (Ki), the Akaishi Mountains (Ak), and the Hakusan mountain range (Ha), which are in the southwestern part of the distribution range. Diversity in the Joshin range (Js) is also high.

### 3.3 Nested clade phylogeographical analysis (NCPA)

All haplotypes were grouped into one 4th level clade (Fig.4). NCPA showed a significant association between genetic and geographic distribution (Fig. 5). There are 5 clades in Hokkaido and 9 in Honshu where a significant tendency ( $P < 0.05$ ) of genealogy and geographic location was suggested by NCPA (Table 3). Clade level 1 in Hokkaido with low significant probability (clade 1-5, 1-8) is also shown for reference. Based on the inference key (Templeton, 2004), the history of *E. nipponica* in the Japanese islands was punctuated by several cycles of allopatric fragmentation followed by range expansion.

### 3.3.1 Distribution transition in Hokkaido

#### (1) Early fragmentation

After migrating into the Japanese islands, and spreading out into areas that include the present distribution ranges, they dispersed into two disjunct refugia during warm interglacials, and divided into two lineages, the western lineage (clade 3-1) and the middle eastern lineage (clade 3-2, which includes the eastern and the northern lineages) (allopatric fragmentation of clade 4-1). Then within the western lineage (clade 3-1), the populations of both Rishiri Island and Rebun Island (clade 2-1) were fragmented from populations in the mainland of Hokkaido.

#### (2) Origin of northern lineage

According to NCPA, the northern lineage (clade 2-4) is inferred to have expanded by contiguous range expansion from other clade 2-3 (these were included in clade 3-2).

#### (3) Recent dispersal and habitat islanding

There are traces that the two lineages, the eastern and the western, which had developed into genetically different populations during disjunct distribution in refugia, later joined together again when they expanded their distribution in the next glacial period (see Section 3.4). As all the populations in Hokkaido have low statistical significance at clade 1, events in the recent period cannot be inferred by NCPA.

### 3.3.2 Distribution transition in Honshu

#### (1) Early fragmentation

A panmictic population at an early glacial period divided into two separate populations of the southern lineage and the northern lineage as a result of isolation in the south refugia and in the north refugia during the following warm interglacial period (allopatric fragmentation of clade 4-2).

#### (2) Range expansion after the early fragmentation

The northern lineage, which is a 2nd level clade belonging to clade 3-3, is inferred to have relatively long distance contiguous range expansion from the Akaishi Mts (Ak) through the Joetsu range (Jy) to the northernmost populations in Honshu (Chokaisan (Ch), the Mahiru Mts (Wa), the Hayachine range (Hy)). The southern lineage has dispersed over some long distance, forming the Myoko range population (clade 2-7), central Mountain population (the Hakusan range (Ha), the Hida Mts (Hi), the Kiso Mts (Ki), Yatsugatake (Yt)) (clade 2-8) and the Joshin range population (Js) (clade 2-9).

#### (3) The second fragmentation, habitat islanding

The northern population (clade 2-5) is fragmented into clade 1-13 and clade 1-14 by allopatric fragmentation. Each of the following populations is inferred as past fragmentation followed by range expansion: the population (clade 2-6) which ranged from a southern part of the northern area to the Akaishi Mts (Ak), the population (clade 2-8) which ranged widely over the central mountainous areas (the Hakusan range (Ha), the Hida Mts (Hi), the Kiso Mts (Ki), Yatsugatake (Yt)), and the Myoko range (My) population. The Joshin range (Js) population (clade 2-9) expanded by restricted gene flow with isolation by distance. In this area, there is no mountain range which constitutes a continuous geographical connection. Instead, relatively low mountains lie scattered separately. So, it seems a reasonable inference.

#### (4) Recent fragmentation, multiple islanding

Only clade 1-21 is statistically significant, and it is inferred by allopatric fragmentation with further fragmentation into each mountain range (the Hida Mts, the Kiso Mts, Yatsugatake).

### 3.4 Secondary contacts

A few pieces of evidence of secondary contacts can be seen in the haplotype distribution. As an example of the inter-lineage secondary contact in Hokkaido, a mixed area of the western lineage and eastern lineage is observed in Nakatoikan, Horonobecho (N44°58'37", E142°02'34") in northern Hokkaido. In Honshu, a mixed area of the NS100 haplotype in the northern lineage and the CH130 haplotype in the southern lineage is observed in part of the Joetsu range (Jy) (N36°53'38", E138°57'01") (Fig. 6). In this mixed distribution area in the Joetsu range (Jy), mixed inhabitation is observed in all 3 populations existing within 410 m altitudinal difference and within 2 km horizontal distance. As an example of intra-lineage secondary contact within the northern lineage, the haplotype NS150 which mainly inhabits the Yakeishi range (Yk) is observed with the haplotype NS170 together inhabiting a part of the Asahi Mts (As) (N44°59'55", E142°01'36") which lie 130 km southwest from the Yakeishi range. Within the southern lineage, the haplotype CH120 which ranges widely over the middle south area of the Hida Mts (Hi-S), Yatsugatake (Yt), the Kiso Mts (Ki) and the Hakusan range (Ha), is found with the haplotype CH110 in the southern area of the Myoko range (My) (N36°47'31", E138°04'10"). At the same time, the haplotype CH110 which ranges over the Myoko range (My) is found in the northern part of the Hida Mts (Hi-N) (around N36°45'42", E137°45'32"). These areas are about 75 km apart and partitioned off by the Fossa Magna with 200–400 m altitude. Also the haplotype CH110 of the Myoko range (My) is also found together with the haplotype CH130 in the western peripheries of the Joshin range (Js) (N36°44'38", E138°33'34"). *Erebia niphonica* is not currently found in the intervening areas between these secondary contact points (Fig. 7).

## 4. Discussion

Inferences of historical processes made by NCPA should be viewed with caution, because the inference keys make qualitative assessments, and do not give a statistical approval to a counter hypothesis (Knowles and Maddison, 2002). Therefore, we will examine below the historical transition of the distribution range of *Erebia niphonica* with NCPA inference as the basis while taking its distribution and geographical characteristics into consideration. We will also discuss the distribution range transition based on the ecological characteristics of *Erebia niphonica*.

### 4.1 Lineage fragmentation in multiple refugia

As can be seen in the haplotype network for NCPA (Fig. 3), there are 2 mutation steps between the western and eastern lineages in Hokkaido, and 3 mutation steps between the northern lineage and southern lineage in Honshu. The first fragmentation in Hokkaido and Honshu can be considered to have taken place roughly at the same time. It is suggested that the isolation period of the two lineages in Hokkaido was relatively shorter than that in Honshu. This is probably because that Hokkaido was located in the north and therefore cooler than Honshu.

Among the three lineages of Hokkaido, the northern lineage is separated from the other lineages by 6 mutation steps (There is one haplotype HE000 between them. But this was found in one sample among many obtained at a certain sampling sites). The isolation period of the northern lineage is inferred to be long, but their current distribution range is re-

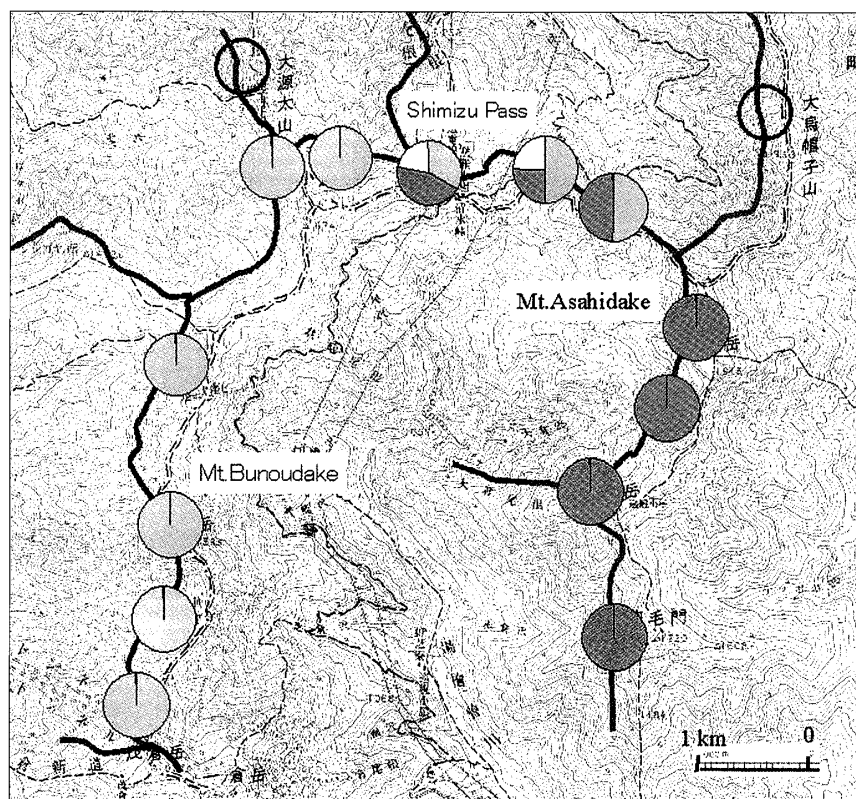


Fig. 6. The northern lineage and southern lineage of Honshu have mixed distribution areas in a part of the Joetsu range (Jy) (N36°53'38", E138°57'01"). Three mixed populations were confirmed to be scattered in the area within 410 m altitude difference and 2 km horizontal distance. Pie charts show the proportions of haplotypes within each population, orange; CH130 (southern lineage), blue; NS100 (northern lineage), white; NS121 (northern lineage). Open circles denote populations without sampled. Heavy line denotes the ridge.

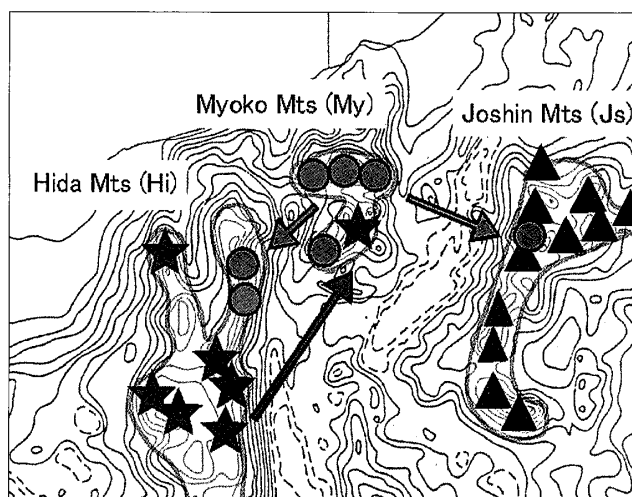


Fig. 7. The mixed ranges of the haplotypes in northern mountainous area of central Honshu are the traces of the secondary contacts of the widely distributed haplotypes. Each mark denotes haplotype, circle; CH110, star; CH120, triangle; CH130. The shaded areas show current distribution ranges. Arrows show the directions of range expansions in the past. Abbreviations denote the range names corresponding to Table 1.



stricted to a localized area in the north, and they coexist with the eastern lineage at many sites in the distribution range. Therefore, there is a possibility that they migrated into Hokkaido in a (later) different era from that in which the other two lineages originated. Recent events in Hokkaido cannot be inferred by NCPA as the statistical significance is low. But the topology of haplotypes (Fig. 4) suggests a possibility that many individual haplotypes were produced in localized areas by the bottleneck effect, while the environmental stresses increased during the recent warm interglacial period.

As the lineage is fragmented into two, the northern and southern ones, in Honshu, it is suggested that there were two refugia during interglacials (Avice, 2000). We have observed in the alpine belt of Honshu that *Erebia nipponica* neither inhabits dry windward grasslands nor expansive grasslands without surrounding forests (Nakatani and Kitagawa, 2000, 2001; Nakatani and Hosoya, 2003). Considering these ecological requirements of the subject species, the important prerequisite for refugia is not only adequate temperature but also humidity adequate to allow coniferous forests and grasslands to grow together in patched patterns. Isolation may have also been taking place in quite dry glacial periods. This situation is apparently similar to the case of *Erebia epiphron* in Europe (Schmitt *et al.*, 2006).

Regarding the two lineages of Honshu, the northern lineage with the haplotype NS100 is scattered over a range of about 280-km between the Akaishi Mts (Ak) and the Iide Mts (Ii). On the other hand, the southern lineage with the haplotype CH120 spreads widely from the Hakusan range (Ha) in the west to Yatsugatake (Yt) in the east with the Hida Mts (Hi) being their main distribution range. These facts suggested that the *Erebia nipponica* populations not only migrated vertically but also dispersed horizontally for long distances through glacials and interglacials. The dispersal seems to be phalanx type (Hewitt, 1996, 2000) as it occurred while keeping uniform genetic characteristics. For *Parnassius smintheus* in the Rocky Mountains, vertical movement was suggested as the main movement (DeChaine and Martin, 2004). This seems to be caused by the difference in the environmental requirements of the subject butterflies and of the available mountain altitudes. While the habitat of *Parnassius* is a dry area at a higher altitude, that of *Erebia nipponica* is in the grasslands in subalpine coniferous forests at a lower altitude. While the Rocky Mountains are made up of mountains higher than 4,000 m, mountains in the Japanese islands are for the most part about 2,500 m high. Thus, it seems that during the warm periods *Erebia nipponica* was unable to move to a suitable environment by moving only upward, and was forced to move along the latitude.

#### 4.2 Primary cause of vicariance of the Honshu northern lineage

The haplotype NS100 of *Erebia nipponica* of the Honshu northern lineage is scattered over a range of about 280-km from the Akaishi Mts (Ak) to the Iide Mts (Ii) (Fig. 2b). The distribution range of *Erebia nipponica* in Honshu is strongly connected with the distribution range of *Abies mariesii*, which is the main constituent tree species in the subalpine coniferous forests (Nakatani and Kitagawa, 2001). In the northern area including the Iide Mts (Ii) the distribution range of *Abies mariesii* and that of *Erebia nipponica* are exclusive to each other, and in the central area including the Akaishi Mts (Ak) they are together. The trunk of conifers is rather weak and breaks easily under lateral pressure. Therefore, along a mountain stream, conifers fall down under the effects of flood and avalanche. A grassland is then formed there. In the grassland, young conifers grow gradually, and the grassland persists along the stream within the forest for some time, until the young conifers grow up. Formation and disappearance of grassland along streams in forest take place repeatedly. Female *Erebia nipponica* from nearby colonies would visit these newly formed grasslands within subalpine coniferous forests, and this could result in the formation of a new popula-

tion. When the mountain is high enough to support an alpine herbaceous zone beyond the timberline, where *Erebia niphonica* breeds stably, it can continue to repeat expansion into or contraction from the grasslands forming and disappearing in coniferous forests. However, in low mountains where conifers cover the top of the mountain, *Erebia niphonica* cannot maintain a continuous population, even if grasslands are formed along mountain streams. Mountains in central Honshu are so high that they go beyond the timberline, and *Erebia niphonica* is distributed where there are conifers. But most of the mountains in northern Honshu are not sufficiently high to support an alpine herbaceous zone, with the result that conifers cover the top of the mountains. Therefore, *Erebia niphonica* cannot occur there. In the northern Honshu population area, *Erebia niphonica* are found only in grassland in the mountains where growth of conifers is suppressed by the severe winter climates, with heavy snow or strong wind. Mountains with altitudes of 2,500 m or higher are scattered like stepping stones in the areas from the Akaishi Mts (Ak) to the Iide Mts (Ii). These mountains are covered with conifers to the summits at present. But in the glacial periods, the timberline was lower, and there were alpine herbaceous zones at the top, in which *Erebia niphonica* could have maintained populations. In the southern Akaishi Mts (Ak) there are several mountains which are covered by conifers to the summits, but have grasslands on steep precipices, in which *Erebia niphonica* is found. These populations could be the last survivors after the grasslands at the top disappeared.

#### 4.3 Genetic diversity

In Hokkaido, the standard diversity index of each regional population is within a 0.50–0.65 range in many areas, and regional differences in the haplotype diversity are small. Populations in the Soya area (So) and the Teshio Mts (Te) have high diversity. This is due to the endemic distribution range of the northern lineage. In Honshu high diversity populations exist in the southwest. It is suggested that the stress of environmental changes was higher in the southwest, that repeated expansion and contraction of distribution ranges took place, and that during these processes haplotypes were fixed by the bottleneck effect. Many of the fixed haplotypes are different from the widely spread haplotype by 1 mutation step, and it may have happened in the recent warm period. On the other hand, the haplotype diversity is low in the populations in the northern area. The northern area consists of relatively low mountains, and it is suggested that populations might have been reduced or even have disappeared by strong selection pressures during the warm period, and that there is a possibility that the populations in the northern area were reconstructed from small numbers of small populations in the next glacial period. The species that were able to survive in refugia in northern Honshu must have been limited to those that had high adaptability to environmental conditions such as temperature and food plants. There are 9 alpine butterflies in the entire island of Honshu. But in the northern Honshu area only *Erebia niphonica* now exists. The strong adaptability of this species, which was mentioned in the Introduction, must have functioned effectively.

The existence of traces of secondary contacts by different lineages, found in Hokkaido and in Honshu, is direct evidence that the *Erebia niphonica* populations underwent repeated fragmentation and dispersal as a result of glacial cycles. It suggests that distribution ranges fragmented into separate areas like islands in the past warm period, in each of which a population with characteristic haplotypes evolved, and that later in the glacial period these populations expanded and came into secondary contact with each other, following which, in the next warm period the distribution ranges have again become islands. The result of NCPA analysis is consistent with such a scenario. These traces of secondary contacts of different lineages strongly support the notion that cycles of fragmentation and dispersal of distribution ranges in the Pleistocene took place in the Japanese islands even on a small scale, just

as proposed for *Parnassius smintheus* in the Rocky Mountains (DeChaine and Martin, 2004). In this study, the sampling locations were much more numerous than those in previous studies and this led to the discovery of the traces of the secondary contacts. It is better to increase sampling locations provided that equal numbers of samples are maintained. This should help a better understanding of phylogeography in future studies.

Regarding many alpine organisms in the Japanese islands, it is proposed that there are two lineages, the northern one which originated in the north and migrated from the Amur region or the Kamchatka Peninsula, and the southern one which originated in the south and migrated through the Korean Peninsula (Saigusa, 2005). In order to take forward phylogeographical study of animals and plants in the Japanese islands in future, a field study including detailed observations of habitats in the northern mountain area of the Korean Peninsula is highly desirable, as well as of those in Sakhalin, the Kuril Islands, and the Amur region in Russia.

### Acknowledgments

Most of the distribution ranges of *Erebia nipponica* in Honshu are located within the Special Protection Zones of National Parks or Quasi-National Parks, and all animals and plants are protected. Also some local governments are protecting this species by designating it as a Natural Monument. Therefore, when we collected samples, permissions were granted by the Ministry of the Environment, by the Agency for Cultural Affairs, by local governments, and by other related organizations. This study was partly supported by grants from the Foundation of Nagano Prefecture for Promoting Science (to T. Nakatani), and Fujiwara Natural History Foundation (to T. Nakatani). We thank Y. Ohshima and M. Kogure for their help in the field study on the Asian continent. We also thank J. Asahi, N. Aso, T. Okamoto, K. Ono, A. Kitagawa, N. Takeuchi, M. Tashita, M. Fukumoto, Y. Hosoya, K. Maruyama, and many other people who helped us to collect samples. We are grateful to Y. Nakamura and Y. Matsuda of Shinshu University School of Medicine for technical assistance. We also thank S. Ueda of Graduate school of Science and Technology, Shinshu University for valuable discussion.

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## 摘 要

第四紀日本列島におけるベニヒカゲの分布変遷史 (中谷貴壽・宇佐美真一・伊藤建夫)

日本列島に分布する高山性生物が、いつどのようなプロセスを経て大陸から渡来したか、また第四紀の気候変動に適応して列島内でどのような分布の変遷を経たかについては、古くから多くの考察がなされてきたが化石の残りにくい生物についての研究は少ない。最近の分子情報に基づく研究では高山植物エゾコザクラとヨツバシオガマに関するものがある。いずれの種も本州に南北2系統が存在しその境界は東北地方にある。このような分布状態を形成したプロセスとして、南系統は本州中部山岳地域を温暖期のレフュジアとして残存し、その後の寒冷期に新たな系統が再度大陸から渡来して北系統となったというシナリオが提唱されている(単一レフュジアモデル)。しかし移動力を有する動物では、気候変動など環境条件の変動によく追従して移動できるため、植物とは違ったシナリオが存在する

可能性がある。本州の複雑な山岳地形やそれに基づく微気象などから、日本列島に複数のレフュジアが存在した可能性が考えられる。すなわち温暖期に複数のレフュジアに避難することで地理的に隔離された結果遺伝的に特化した個体群が、その後の氷河サイクルに反応して分布を拡大・縮小することで複数の系統として今日に至っているというシナリオである(複数レフュジアモデル)。

このような可能性を調べるためには、調査対象生物は次のような特性を備えていることが必要である。(1) 環境の変化に反応して好適な環境へ容易に移住できる移動能力を備えていること。(2) 個々の個体は通常はあまり移動を行わず、生まれた集団にとどまっていることで、個体群としては系統地理的情報を継承していること。(3) 生息可能な環境が地理的に狭くても継続して生息できること。(4) 離散的ではあっても現在広域に分布していること。

ベニヒカゲはこれらの条件を備えており、第四紀氷河サイクルの気候変動によく追従して分布を拡大・縮小すると共に、その歴史の変遷の履歴を集団の遺伝的構造に残している可能性が高いと期待される。

ミトコンドリア DNA (mtDNA) の *ND5* の一部 (432 bp) および *COI* の一部 (510 bp) の塩基配列を決定し、これらを結合した配列 (942 bp) に基づいてハプロタイプを検出した。分析したサンプルは北海道産 119 個体群 147 個体、本州産 170 個体群 276 個体、合計 289 個体群 423 個体である。その他参考用としてモンゴルハンガイ山脈産、ロシアアムール地域産各 2 個体を用いた。

検出されたハプロタイプは、北海道産 22、本州産 25、合計 47 タイプであり、統計的最節約法により系統関係を推定すると、大陸産、北海道産、本州産の 3 つのクレードに分岐し、それらの集団間の塩基置換は非常に大きく、一つのネットワーク樹に結合しない (Fig. 3)。サンプル収集地点を北海道 12、本州 17 の地域に分けて、AMOVA により分散分析を行うと地域間の分散比率が有意に大であり、遺伝子流動が制限され集団間に地理的な隔離があることを示唆している (Table 2)。Nested Clade Phylogeographical Analysis (NCPA) により集団に起こった過去の遺伝的イベントを解析した。Inference key (Templeton, 2004) によると、日本列島におけるベニヒカゲの分布変遷の歴史は、異所的分断と連続的な分布の拡張または分散の複数回の繰返しであることが示唆される (Table 3)。

本州集団の 4-2, 3-4, 2-8, 1-21 の各クレードが全て統計的に有意と推定されているので、これらについて歴史的プロセスを追ってみると (Table 3)、地理的に分化していない単一の遺伝的特性を有する集団が、温暖期間氷期に異所的な 2 集団に分断される (クレード 4-2)。その内クレード 3-3 の集団は本州東北部に主として分布する北方系統であり、クレード 3-4 は中部地方に分布する南方系統である。次の氷河期に北方系統 (クレード 3-3) は range expansion、南方系統 (クレード 3-4) は dispersal と推定されるように両系統は共に分布を拡張した。南方系統では分散に続いて、クレード 2-7, 2-8, 2-9 において過去の分断とそれに続く分布の拡張、または地理的距離により制約された遺伝子流動が生じた。クレード 1-21 では、引き続いて最新の分断が生じ、現在の分布域にみられるような分断分布が成立した。

地理的に隔離された二つの集団間では遺伝的に異なるハプロタイプが固定される。その後の環境条件改善に適応して分布が拡大すると、再び集団が会って (二次的接触) 二つのハプロタイプの混生状態が生じる可能性がある。筆者らは二次的接触の痕跡と思われる広域分布型ハプロタイプの混生地を発見することができた。すなわち次の二箇所である。(1) 北海道西部系統の分布域である幌延町中間間に東部系統のハプロタイプ (HD000) が分布する。(2) 谷川連峰の一部で本州北部系統と南部系統が分布する (Fig. 6)。また同系統内の広域分布型ハプロタイプの二次的接触跡として次のような場所がある。(3) 飛騨山脈の白馬岳以北に妙高山系のハプロタイプ (CH110) が分布し、逆に妙高山系の一部に飛騨山脈のハプロタイプ (CH120) が分布する。(4) 上信地域の岩菅山に妙高山系のハプロタイプ (CH110) が分布する。これらは温暖期に飛騨山脈、妙高山系、上信山系に隔離された集団内で、それぞれ別のハプロタイプが固定され、その後の寒冷期に分布を拡大した結果二次的接触を生じ、その後の温暖期に再び隔離され相手側の集団内に一部の個体群が残存している状態であると解釈される (Fig. 7)。(5) 朝日連峰南西端に焼石山系のハプロタイプ (NS150) が分布する。

NCPA による解析と二次的接触の具体的事例から、次のような分布変遷プロセスが示唆される。つまり、温暖な間氷期には北海道、本州ともに 2 ケ所のレフュジアが存在し、長期にわたり隔離された結果、北海道東部・西部、本州北部・南部のそれぞれ 2 系統の広域分布型ハプロタイプが生じた。次の寒冷期に分布を拡大した結果二次的接触を行い、後氷期になって再び分断され現在に至っている。二次的接触の痕跡の発見は、氷河サイクルに適応して分布拡大、縮小を繰返した直接的な証拠であると解釈さ

れる。

日本列島における高山性生物の由来については、大陸からの複数回にわたる渡来というシナリオだけではなく、列島内の複数地域がレフュジアとして機能することで、複数の系統に分岐したケースも多いと思われる。本研究では氷河サイクルに適応してベニヒカゲ集団が分布の分断・拡大を繰り返した証拠となる二次的接触の跡を発見したが、これは現在の分布域を広くカバーする地域からサンプルを収集した成果であって、他の生物でもサンプル収集の密度を高めれば、二次的接触の跡が見つかる可能性が高いであろう。

(Accepted December 31, 2006)